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Structural features of the cell-wall polysaccharides of *Asparagus officinalis* seeds

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ABSTRACT

The fine structure of a β-(1→4)-linked glucomannan from *Asparagus officinalis* has been determined by n.m.r. analysis of the oligosaccharides obtained by acidic and enzymic hydrolyses. Cleavage of the glucomannan with β-D-mannase from *Aspergillus niger* and purification by h.p.l.c. gave oligosaccharide fractions that contained Man (mannose), GlcMan (β-glucopyranosylmannose), Man_2, Glc_2Man, and Glc_3Man as the major components. Simulated digestion of a polymer composed of randomly distributed monomers with the same Glc:Man ratio as glucomannan from *A. officinalis* led to the same polysaccharides. The random distribution of the monomers of glucomannan from *A. officinalis* was corroborated by the diffraction diagram of the raw flour, which indicated that the "*in situ*" glucomannan was amorphous, whereas both cellulose and mannans are crystalline.

INTRODUCTION

Cell-wall polysaccharides of seeds often contain mannose residues. Mannans and glucomannans have been extracted from the seeds of monocot species and galactomannans have been found in leguminous seed endosperms[1]. Most present knowledge of the structure of these complex macromolecules is based on the results of methylation analysis and such associated techniques as periodate oxidation. However, these methods give average structures, and little information on the sequence of the residues has been obtained. Methylation analysis data for the glucomannan from *Asparagus officinalis* suggested a β-(1→4)-linked backbone that contained almost equal proportions of D-glucopyranosyl and D-mannopyranosyl residues, but their distribution was uncertain[2]. Recently, ^{13}C-n.m.r. spectroscopy, which can indicate the kinds and the proportions of different residues in polysaccharides, has been used to investigate glucomannans from the bulbs of *Lilium auratum*[3] and *Aloe vahombe*[4]. Chemical hydrolysis of these glucomannans, followed by isolation and n.m.r. spectroscopy of the resulting oligosaccharides, has shed light on the fine structure. Enzymic analysis of glucomannans with β-D-glucanases and β-D-mannanases has been reviewed[5]. However, McCleary and Matheson[6] have shown that, for glucomannans, transglycosylation can be significant, which limits the potential of these enzymes for the determination of fine structure.

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We now report a reinvestigation of the polysaccharides from the cell walls of the endosperm of *A. officinalis*, using ^{1}H- and ^{13}C-n.m.r. spectroscopy of the products released by the actions of β-D-glucanase, β-D-mannanase, and mild acid.

RESULTS AND DISCUSSION

Native polymer. — On gel-permeation chromatography of the glucomannan in Tris buffer, a single peak was eluted from Sephadex G-150, just after the void volume, whereas the carbohydrates were retained on Sephadex G-200, indicating molecular weights in the range 150 000–200 000. The X-ray diffraction diagram (Fig. 1) indicated some crystallinity since three distinct peaks were resolved. In polarised light, some small needles similar to those of mannan I polymorphs[7] were observed. In constrast, not a single peak was detected in the diffraction diagram of the raw flour, indicating that, *in situ*, the glucomannan was in an amorphous state.

The ^{1}H- and ^{13}C-spectra for a solution of the glucomannan in 0.5M NaOD are shown in Fig. 2. The ^{13}C-n.m.r. assignments were based on comparison with data for the glucomannans from *L. auratum*[3] and *A. vahombe*[4], whereas the partial ^{1}H-n.m.r. assignments were obtained from the COSY spectrum. In agreement with the results from chemical analysis[2], these data, particularly the chemical shifts of the C-4 resonances[5], indicated that the polymer consisted of 4-linked glucose and mannose residues in the ratio 2:3 (*cf.* 1:3 in the above glucomannans[3,4]). The β-pyranose form was assigned to the glucose residues from the chemical shifts of the H-1 and C-1 resonances and the $J_{1,2}$ values compared with those of methyl α- and β-D-glucopyranoside[8–10]. Unambiguous identification of the anomeric form of the mannose residues was not possible from the n.m.r. data alone. Signals at 2.17 p.p.m. in the ^{1}H-n.m.r. spectrum suggested that the polymer was partially acetylated. The corresponding signals in the ^{13}C-n.m.r. spectrum could not be detected, but the S/N ratio was poor (< 3) due to low solubility. Partial acetylation of the glucomannan from *L. auratum*[3] has been reported.

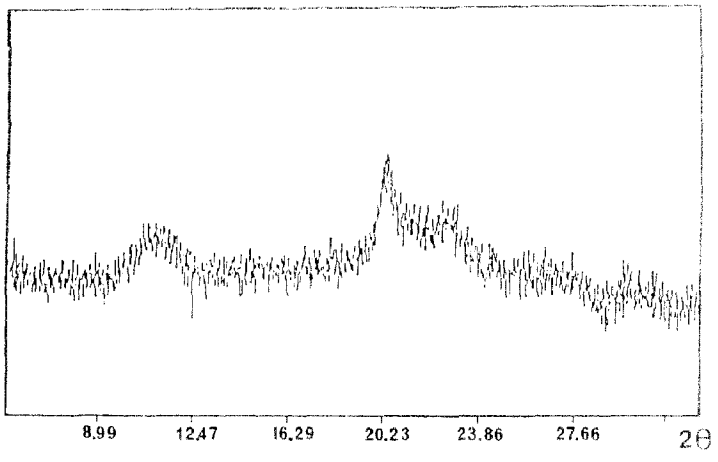


Fig. 1. X-Ray diffraction spectrum of the native glucomannan.

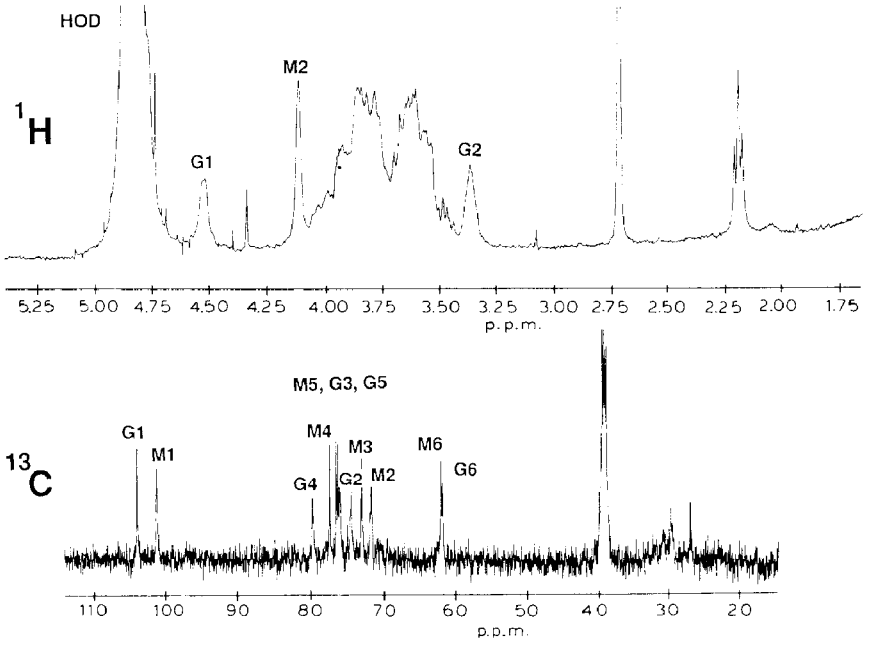


Fig. 2. 400-MHz ^{1}H-n.m.r. and 100-MHz ^{13}C-n.m.r. spectra of a solution of the glucomannan in 0.5M NaOD (internal Me_2SO, δ_C 39.5, δ_H 2.72 p.p.m.); G1 connotes H-1 of glucosyl residues in the ^{1}H spectrum and C-1 of glucosyl residues in the ^{13}C spectrum, *etc.*

Acid hydrolysis. — The main monosaccharides formed on hydrolysis were glucose and mannose (detected by h.p.l.c.). The best yield (~80%) of monomers was obtained on hydrolysis with M sulfuric acid for 2 h at 100°. The Glc:Man ratio increased with increasing acidity, which suggests that mannosyl linkages might be cleaved more easily than glucosyl linkages. However, some mannose molecules might be lost when the hydrolysis was carried out under strongly acidic conditions. Incubation of the glucomannan in 0.05M acid at 100° rapidly hydrolysed the oligosaccharides formed (100% after 6 h). However, after hydrolysis for 1 h, gel-permeation chromatography of the hydrolysate on Bio-Gel P-2 gave four fractions (*A–D*) which represented 38, 27, 23, and 12%, respectively, of the solubilised sugars. Peak *A* was eluted just after the void volume of the column, indicating d.p. > 10.

The oligosaccharides were subjected to n.m.r. spectroscopy. The signals for C-1 of reducing α-glucose, reducing α- and β-mannose, reducing β-glucose, non-reducing β-mannose, and non-reducing β-glucose residues were distinguished readily in the 100-MHz ^{13}C-n.m.r. spectra at 93, 95, 97, 101, and 104 p.p.m., respectively. In contrast, the signals for H-1 of reducing α-glucose, reducing α- and β-mannose, non-reducing β-mannose, reducing β-glucose, and non-reducing β-glucose residues appeared in overlapping regions of the ^{1}H-n.m.r. spectra at 5.25, 5.21, 4.94, 4.77, 4.69, and 4.55 p.p.m., respectively. However, due to mutarotation, the relative intensities of the signals for H-1 of mannose (α > β) and glucose (β > α) are useful criteria. The $J_{1,2}$ values (3.8 Hz for H-1 of α-Glc*p* and 1.5 Hz for H-1 of β-Man*p*) and T_1 values[11] (see below) are also diagnostic.

TABLE I

^{1}H- And ^{13}C-n.m.r. analysis of fractionated oligosaccharides obtained by hydrolysis of glucomannan

Hydrolysis fractions	*Average d.p.*	*Ratio of non-reducing sugars (Glc:Man)*	*Ratio of reducing sugars (Glc:Man)*
Native glucomannan	1000 ± 100	0.66	
Acidic hydrolysis			
A	>10	0.75	0.25
B	5	0.78	0.23
C	5	0.96	0.16
D	1.5	0.89	0.41
β-D-Mannanase			
1	1.2	0.25	0.03
2	2	10	0.02
3	2	0.47	0.06
4	2.5	2.2	0
5	2.5	3	0.17
6	3.5	5.3	0.09
Endo-glucanase			
1	1		0.33
2	2	>13	0
3	2.1	1	0.89
4	2.1	0.26	3.2
5	2.8	1.26	0.66

Thus, based on the ^{1}H- and ^{13}C-n.m.r. data, the relative proportions of mannose and glucose amongst the reducing and non-reducing sugars, as well as the average d.p., were established for fractions *A–D* (see Table I). Fraction *A* had d.p. 10, and both the Glc:Man ratio and the n.m.r. spectra were almost identical to those of the native glucomannan, suggesting that the distribution of mannose and glucose in the latter must be statistical. The expected mixtures of oligosaccharides were obtained for all fractions, although the non-reducing Glc:Man ratio was slightly higher than that of the native polymer. Mannose was the most abundant reducing sugar in each fraction, which implies that the glycosidic linkage of this residue is more labile than that of glucose.

Enzymic hydrolysis. — Treatment of the glucomannan with *β*-D-mannanase and *β*-D-glucanase rapidly released oligosaccharides (100% solubilisation after 3 h). The oligosaccharides were then gradually cleaved. After 20 h with *β*-D-glucanase, the polymer was hydrolysed completely, whereas the *β*-D-mannanase gave only 35 reducing sugar equivalents for 100 soluble sugar units. These data accord with the findings of McLeary and Matheson[6], and suggest that the endo-*β*-D-mannanase from *A. niger* requires at least 4 binding sites. Short incubations (2 h) followed by gel-permeation chromatography on Bio-Gel P-2 indicated that the carbohydrates released had d.p. <4. H.p.l.c. (Fig. 3) gave 6 fractions which corresponded to mono- (1), di- (2 and 3), tri- (4 and 5), and tetra-saccharides (6). The ^{1}H- and ^{13}C-n.m.r. data of fractions 1–6 are given in Table I.

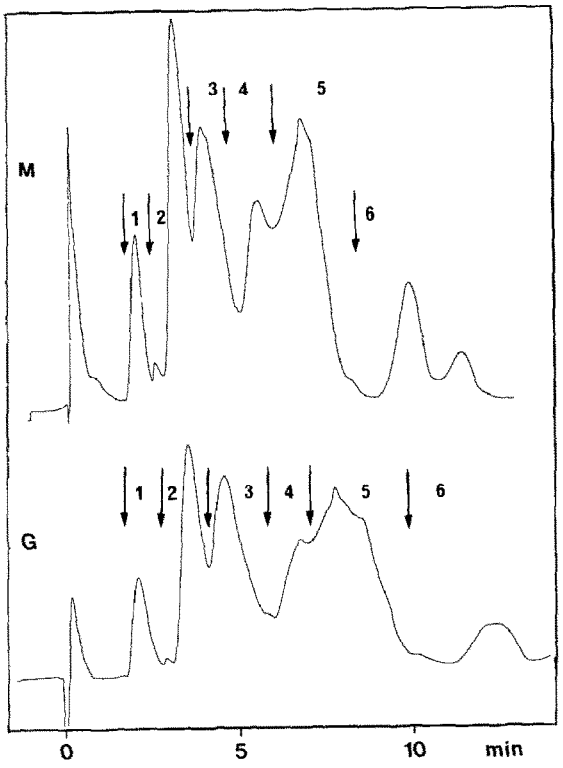
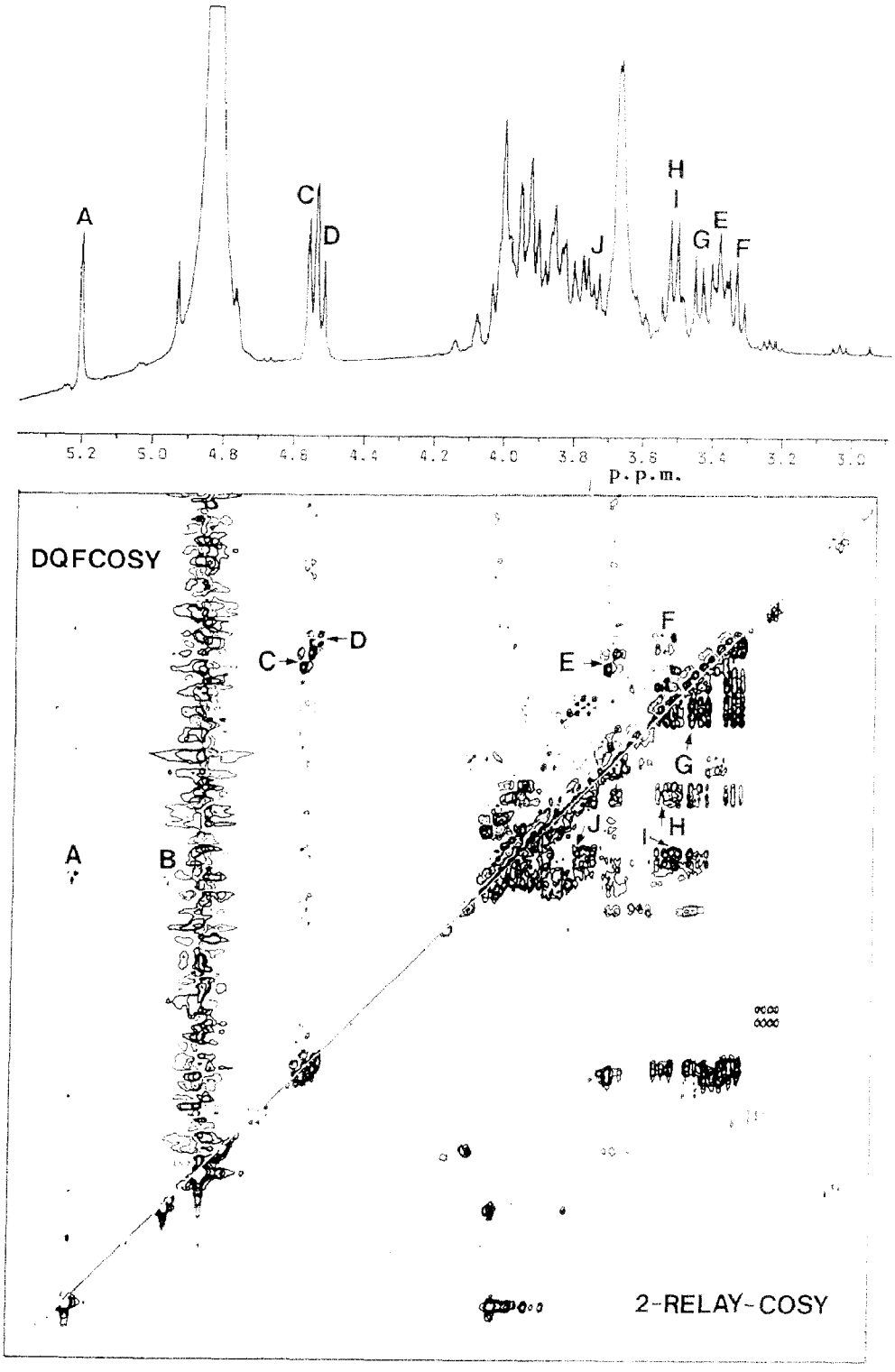


Fig. 3. H.p.l.c. of the oligosaccharides produced on hydrolysis of the native polymer by glucanase (G) and mannanase (M) preparations. Fractions collected are shown with arrows.

Hydrolysis of the glucomannan with β-D-mannanase gave several fractions that were amenable to analysis, as the reducing sugar was almost exclusively mannose. The ^{13}C-n.m.r. spectra of the disaccharides were assigned by comparison with literature data[3], and those of the oligosaccharides by comparison with the spectrum of the related disaccharide. Thus, comparison of the spectrum of β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-Man*p* (Glc_3Man) with that of β-Glc*p*-(1→4)-Man*p* (GlcMan) indicated that two 4-linked glucosyl residues are also present. The glycosylation shifts for the glucose and the mannose residues were close to those predicted by Shashkov *et al.*[12] (β-effects: +6.8 ±0.7 p.p.m. for C-1′ (glycon) and +9.8 ±1.4 p.p.m. for C-4 (aglycon): γ-effects: −1.3 ±0.4 p.p.m. for C-3 and −1.2 ±0.7 p.p.m. for C-5) for (1→4)-linked disaccharides with no equatorial β-hydrogens on the aglycon. The corresponding ^{1}H-n.m.r. spectra were assigned partially from the corresponding 2D double-quantum-filtered phase-sensitive COSY[13] and relayed COSY[14-16] spectra. In Fig. 4, both the DQFCOSY (upper left) and the 2-RELAY-COSY (lower right) spectra of Glc_3Man are given. Most of the H-1,2, H-2,3, and H-3,4 cross-peaks were visible in at least one of these 2D spectra, and the corresponding assignments have been indicated on the 1D spectrum. As with the ^{13}C-n.m.r. spectra, the relative intensities of the various peaks were those expected (2:1 for the signals of the branched glucosyl residues compared to


A
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H
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those of the non-reducing terminal residue). Assignment of the H-1 signals of the reducing mannose residues was based on the values of the corresponding T_1 parameters (1.5, 0.9, and 0.6 s for H-1 of α-Man*p*, β-Man*p*, and β-Glc*p*, respectively, in Glc_3Man) as well as their relative intensities. For α-mannose, H-1 has a much longer T_1 than that of β-mannose[11].

In a similar manner, the following oligomers could be identified: GlcMan, β-Glc*p*-(1→4)-Man*p*; Man_2, β-Man*p*-(1→4)-Man*p*; and Glc_2Man, β-Glc*p*-(1→4)-β-Glc*p*-(1→4)-Man*p*. The ^{13}C- and ^1H-n.m.r. data for thse oligosaccharides are collected in Tables II and III, respectively. The $^3J_{H,H}$ values were those expected for gluco- and manno-pyranose residues in the $^4C_1$ conformation[17]. As expected[18], the ^1H-n.m.r. glycosylation shifts were ~ +0.15 p.p.m. for the resonances of H-3 and H-4 of the aglycon, and ~ −0.15 p.p.m. for the H-1 resonances. Finally, the ^1H data of Man_2 agree well with those reported[19].

It should be emphasised that the oligosaccharide fractions were mixtures and only the major components could be identified by n.m.r. spectroscopy. Similar oligosaccharides were obtained from konjac glucomannan by hydrolysis with β-D-mannanase from *Penicillium purpurogenum*[20].

Enzymic hydrolysis of glucomannan with endo-glucanase was much less selective and gave mixtures of oligosaccharides that contained both mannose and glucose as the reducing sugar. However, the major components of fractions 2 and 4 could be identified as GlcMan and β-Man*p*-(1→4)-Glc*p* (ManGlc), respectively, and the n.m.r. data for this latter disaccharide are given in Tables II and III. For the hydrolysis with β-D-mannanase, the main component of fraction 4 was Glc_2Man, whereas GlcMan and Man_2 were eluted in fractions 2 and 3, respectively. This result suggests that saccharides with reducing glucosyl residues were more strongly retained on the Aminex column.

In a study of the patterns of action and substrate-binding requirements of β-D-mannanase, McCleary and Matheson[6] proposed a model (Fig. 5A) with 5 binding-subsites based on the favoured conformation of the (1→4)-β-D-linked mannan chain, which is a flat ribbon with a two-fold axis, that places neighbouring hydroxymethyl groups on opposite sites of the ribbon[21-23]. A similar conformation is expected for glucomannans, and it has been shown by X-ray and electron diffraction studies[7,24] of glucomannans and molecular modelling[25] of Man_2 and GlcMan that β-D-glucose residues isomorphously replace those of β-D-mannose. In the above, rings B and D are thought to bind through the hydroxymethyl groups (and possibly the oxygen ring), and rings A, C, and E through HO-2, and/or HO-3. Of the three rings A, C, and E, for which the HO-2,3 edge of the ring differs in stereochemistry at HO-2 depending on which residue (Glc or Man) is present, only sites C and E were expected to have a strong

Fig. 4. 400-MHz 2D-N.m.r. spectra of a solution of Glc_3Man in D_2O (~ 10 mg/0.5 mL) and the corresponding 1D spectrum. The following cross-peaks are labelled in both the 1D and 2D spectra: DQFCOSY, A, H-1,2 for α-Man*p*; B, β-Man*p*; C, β-Glc*p* (2nd and 3rd residues); D, β-Glc*p* (4th residue); E, H-2,3 for β-Glc*p* (2nd and 3rd residues); F, β-Glc*p* (4th residue); 2-RELAY-COSY, G–J, H-3,4, H-5,6a, H-5,6b, and H-6a,6b (4th residue).

TABLE II

100 MHz ^{13}C-n.m.r. chemical shift data for glucomannan oligomers in D_2O

Atom	*ManGlc*	*Man_2*	*GlcMan*	*Glc_2Man*	*Glc_2Man*
Reducing residue					
C-1	93.41	95.35	95.33	95.34	95.30
	97.39	95.23	95.19	95.18	95.16
C-2	72.71	71.76	71.83	71.87	71.84
	75.37	72.17	72.28	72.32	72.28
C-3	72.88	70.48	70.54	70.49	70.48
	76.18	73.22	73.32	73.23	73.25
C-4	80.41	78.36	78.47	78.36	78.33
	80.29	78.04	78.09	77.96	77.96
C-5	71.51	72.43	72.59	72.61	72.58
	75.81	76.32	79.49	76.47	76.45
C-6[a]	62.50	62.55	62.23	62.14	62.13
	61.82	62.04	61.88	61.83	61.81
	61.71			61.53	61.44
2nd Residue[b]					
C-1	101.63	101.70	104.23	104.11	104.11
C-2	72.73	72.07	74.74	74.51	74.47
C-3	74.39	74.35	77.57	76.39	76.35
C-4	68.22	68.24	71.11	80.08	80.02
C-5	78.00	77.96	77.06	75.60	75.58
C-6[a]	62.50	62.55	62.23	62.14	62.13
	61.82	62.04	61.88	61.83	61.81
	61.71			61.53	61.44
3rd Residue[b]					
C-1				103.97	104.00
C-2				74.71	74.45
C-3				77.05	76.36
C-4				71.02	79.98
C-5				77.55	75.56
C-6[a]				62.14	62.13
				61.83	61.81
				61.53	61.44
4th Residue[b]					
C-1					103.96
C-2					74.69
C-3					77.04
C-4					71.02
C-5					77.54
C-6[a]					62.13
					61.81
					61.44

[a] Primary hydroxyl carbons unassigned. [b] Signals for the 2nd and 3rd residues of Glc_2Man may be interchanged.

TABLE III

400-MHz ^{1}H-n.m.r. data[a] for solutions of di- and oligo-saccharides in D_2O

Atom	*ManGlc*	*Man_2*	*GlcMan*	*Glc_2Man*	*Glc_3Man*
Reducing residue					
H-1	5.247 (3.8)	5.206 (3.4)[b]	5.209 (1.7)	5.210 (1.2)	5.203 (4.0)[b]
	4.686 (8.0)	4.935 (2.4)[b]	4.939 (2.4)[b]	4.941 (2.4)[b]	4.931 (2.6)[b]
H-2	3.592 (d, ~8)		4.011	4.010	4.008
	3.310 (9.1, 8.1)		4.011	4.019	4.088
H-3	3.883 (10, 9)				
	3.684 (~t, ~8)	3.797		3.819	
H-4	3.690 (~t, ~8)				
	3.603				
2nd residue					
H-1	4.775 (2.8)[b]	4.763 (3.9)	4.534 (8.0)	4.560 (8)	4.551 (8)
			4.527 (7.9)		
H-2	4.088 (6.5)	4.088	3.345 (9.3, 8.0)	3.388 (~t, ~8)	3.385 (~t, ~8)
			3.335 (9.6, 7.9)	3.378 (~t, ~8)	
H-3	3.681 (~d, ~8)	3.682 (9.3, 3)	3.539	3.682	3.679
H-4	3.605 (~t, ~8)	3.594 (t, 9.5)	3.433 (~t, 9.4)	3.597 (~t, 9.7)	
			3.432 (~t, 9.4)	3.592 (~t, 9.7)	
H-5		3.455	3.539		

TABLE III (continued)

Atom	*ManGlc*	*Man_2*	*GlcMan*	*Glc_2Man*	*Glc_3Man*
H-6a		*3.758*	*3.756* *(12.4, 5.9)*		
			3.846		
H-6b		*3.977*	*3.960*		
			3.994		
3rd residue					
H-1				4.533 (7.8)	4.551 (8)
H-2				3.340 (9.4, 7.8)	3.385 (~1, ~8)
H-3				3.538 (~1, 9.1)	3.679
H-4				3.440 (~1, 9.2)	
4th residue					
H-1					4.525 (8.0)
H-2					3.335 (9.2, 8)
H-3					3.527 (~1, 9.2)
H-4					3.432 (~1, 9.0)
H-5					3.505
H-6a					3.755 (12.6, 6)
H-6b					3.946

[a] Chemical shifts in p.p.m. (*J* in Hz) from 1D spectrum (± 0.5 Hz/pt.); in the case of multiplicity analysis from the 2D spectrum, ±2.5 Hz/pt. [b] $\Delta\nu^{1/2}$ in Hz.

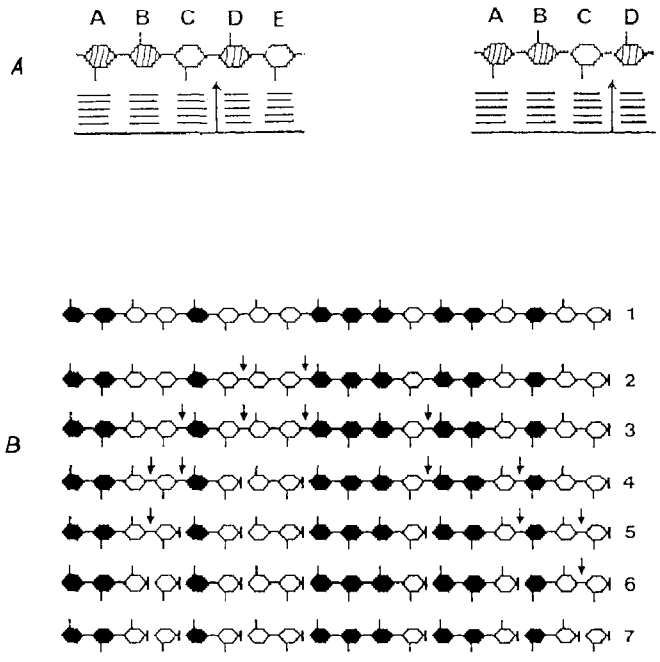

Fig. 5. *A*, Schematic representation of 5 and 4 subsite-binding models for the β-D-mannanase–glucomannan interaction: empty and shaded cycles represent mannose and either mannose or glucose residues, respectively; the projection indicates the orientation of the $-CH_2OH$ group. *B*, Simulation of the digestion by β-D-mannanase of a molecule of glucomannan that contains 18 monomers randomly distributed; five successive steps (2–6) of the digestion (arrows) with random distribution of the enzyme are indicated; 7: hydrolysis end products.

preference for mannose. Futhermore, the existence of a 4 binding-subsite model was evoked.

The isolation of significant proportions of monomers as well as Glc_2Man and Glc_3Man is not compatible with the 5 binding-subsite model proposed for salep glucomannan[6]. However, the 4 binding-subsite model (Fig. 5A) would explain the products of enzymic hydrolysis obtained from *A. officinalis*. It is possible that *A. niger* β-D-mannanase might react with glucomannans through both 4 and 5 binding-subsite interactions. The predominant pathway of hydrolysis might be determined either by the glucose content or the type of distribution of monomer units (statistical or by blocks) in glucomannans. The Glc:Man ratio of glucomannan from *A. officinalis* (2:3) is much higher than that of salep glucomannan (1:3).

In order to test these hypotheses, the digestion of glucomannan with randomly distributed monomer units with a fixed, Glc:Man ratio of 0.67 was simulated under the conditions of the 4 binding-subsite model (see Experimental and Fig. 5B). The same fragments in similar proportions (Fig. 6) were obtained by this simulated digestion of a random polymer as for the digestion of glucomannan from *A. officinalis* by *A. niger* β-D-mannanase (Fig. 3). The proportions of manno-oligosaccharides were low, the absence of the tetrasaccharide being noteworthy. Low quantities (4%) of higher

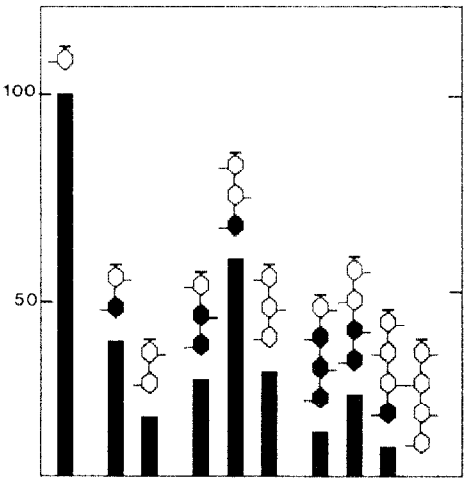

Fig. 6. Count of the various oligomers obtained by the simulated digestion of a glucomannan molecule that contains 1000 randomly distributed monomers with a Glc:Man ratio of 0.7.

oligomers ($4 < d.p. < 8$) were present but are not represented in Fig. 6. Thus, all the evidence points to a random distribution of the monomer units in glucomannan from *A. officinalis*. The results of the present study corroborate those of Chanzy *et al.*[7] who reported poor crystallinity for most samples of glucomannan, whereas both cellulose and mannans are well-crystalline.

EXPERIMENTAL

Preparation of polysaccharides. — Ground seeds of *A. officinalis* glucomannans were stirred successively with ethanol at 80° (3 × 15 min), cold water (24 h), and boiling water (2 × 2 h). The insoluble matter was then extracted with 2M NaOH (2 × 48 h). The supernatant solution was neutralised with HOAc, and the polysaccharides were precipitated with ethanol, dialysed against water, redissolved in 2M NaOH, and treated with Fehling's solution. The complex was recovered by centrifugation, dissolved by the addition of 2M HCl, precipitated with ethanol, dialysed, and freeze-dried. The product was ground in a mortar to give the glucomannan as a fine white powder (750 mg from 1 g of seeds).

Purification of the glucanase. — A commercial mixture (from Nova) that contained mainly an endo-polygalacturonase and an endo-β-D-glucanase was desalted by chromatography on Sephadex G25, then applied to a column (1.8 × 20 cm) of CM Sepharose CL6B equilibrated with 0.002M acetate buffer (pH 4.7). The endo-β-D-glucanases were not retained on the column, and the solution thus obtained was reduced to a small volume by ultrafiltration through an Amicon PM10 membrane, applied to a column (1.8 × 80 cm) of Sephacryl S200, and eluted with 0.1M acetate buffer (pH 4.7).

Hydrolysis of polysaccharides. — Enzymic hydrolyses were carried out in 0.05M acetate buffer (pH 4.7) at 37°. The reaction was stopped by addition of 50 μL of 7.5M

NaOH. Reducing sugars were estimated by the Nelson–Somogyi method and total carbohydrates with anthrone. β-D-Mannanase (Megazyme) from *A. niger* was obtained from Dr. B. V. McLeary and β-D-glucanase was prepared as described above.

Gel-permeation chromatography. — Native glucomannan (50 mg) was dissolved in 2M NaOH (10 mL), the pH was lowered to 9.0 with HCl, and the volume was adjusted to 20 mL with 0.10M Tris buffer (pH 9.0). A portion (2 mL) of the solution was then applied to columns (0.5 × 100 cm) of Sephadex and eluted with Tris buffer. The carbohydrate contents of fractions (1.5 mL) were assayed with anthrone. Carbohydrates released from the native polymer by mild acid hydrolysis were fractionated by chromatography on columns (1.8 × 80 cm) of Bio-Gel P-2.

H.p.l.c. — Carbohydrate solutions were filtered through Millipore membranes (0.45 μm) and injected into a Spectra Physics liquid chromatograph (Model SP8750) equipped with an Aminex column (220 × 4.6 mm) and a guard column (15 × 4.6 mm). Sugars were eluted with acetonitrile–water (3:1) and detected with a Spectra Physics SP 6040 refractive index detector connected to an SP 4290 integrator.

Digestion program. — The program modelled both the glucomannan molecule and the digestion by β-D-mannanase. The construction of the glucomannan molecule consisted of creating a random chain of glucose and mannose residues in the ratio 2:3. The length of the chain is a variable (18 in the schematic digestion of Fig. 5B, 1000 in the example in Fig. 6). The reducing sugar was always a mannose residue. The enzyme hydrolyses the molecule in a random manner, but the linkage cleaved must be at least three units from the terminal non-reducing residue. Hydrolysis always produces a reducing mannose residue. Two enzyme molecules can react simultaneously at a close distance, leading to monomer units. However, as soon as the bond is cleaved, the restriction regarding the distance from the terminal unit is operative, implying that trimers are not cleaved. On each loop of the digestion, the number and the type of fragments are memorised. This distribution of hydrolysis products may be visualised in the form of a theoretical chromatogram such as that of Fig. 6. This program was written in Basic on an Amstrad CPC 6128.

N.m.r. spectroscopy. — A Bruker AM-400 spectrometer operating in the F.t. mode at 400.13 MHz for ^1H and 100.57 MHz for ^{13}C was used. Samples were dissolved in D_2O (10–15 mg in 0.5 mL with 5-mm tubes). Me_2SO was the internal reference (δ_C 39.5, δ_H 2.72). The spectral window for the ^1H-n.m.r. spectra was 10 p.p.m. for 16k data points with a pulse width of 8 μs (45°), an 8-s delay between each scan, and an acquisition time of 2 s. ^{13}C-N.m.r. spectra were recorded with complete proton decoupling and a pulse width of 8 μs (60°). The acquisition time was 1.11 s with a 7-s delay between each scan. Proton spin–lattice relaxation times were measured using the inversion recovery method (180°–τ–90°) with 12 τ values. The peak intensities were measured as a function of the delay time τ and data were processed with the Bruker T_1 routine.

Double-quantum-filtered phase-sensitive COSY experiments[13] were performed using a (90°)–(t_1)–(90°)–(FID, t_2) sequence. The spectral width in F1 and F2 was 1228 Hz; the number of data points in F2 was 1024, and 512 increments were recorded. The

90° pulse was 21 μs and the total acquisition time was 16 h. Before Fourier transformation, the data were multiplied with a $\pi/2$-shifted squared sine bell. Zero filling was applied in F1. The relayed COSY experiments were performed according to the method of Eich *et al.*[14] and the phase-cycling scheme was that reported by Bax and Drobny[15]. The mixing times[16] for the first and second relays were 70 and 30 ms, respectively.

X-Ray diffraction. — An X-ray powder diffractometer operating at 45 kV and 30 mA was used following the transmission Debye–Scherrer method. The X-ray radiation was selected by a Guinier monochromator on $\lambda(\text{Cu-}K_\alpha) = 0.15405$ nm and detected by a scintillation counter with a 1-mm slit. The angular scanning velocity was chosen at 0.5° 2θ/mn with 10 mm/mn chart speed.

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